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Mammary Involution in Dairy Animals

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Lifetime milk production is maximized when dairy cows are pregnant during approximately 70% of each lactation. Between lactations, a nonlactating period is necessary for optimal milk production in the succeeding lactation. With cessation of milking, alveolar structure is largely maintained and little or no loss of cells occurs. However, increased apoptosis and cell proliferation, relative to that in lactating glands during the same stage of gestation, suggest that a nonlactating period serves to promote cell turnover prior to the next lactation. Even in the absence of pregnancy, mammary involution in dairy animals occurs at a slower rate than in rodents; alveolar structure is maintained for several weeks and lactation can be reinitiated after four weeks or more of involution. Although apoptosis appears to be initiated within a similar time frame to that in rodents, the maximum proportion of apoptotic epithelial cells appears to be lower than in rodents, and apoptosis may be accompanied by an initial increase in cell proliferation. The ability to manipulate apoptosis and cell proliferation during the nonlactating period and during lactation is expected to provide enormous benefits to the dairy industry.

KEY WORDS: Mammary involution; cow; goat; sheep; cell turnover; apoptosis.

INTRODUCTION

Cessation of milking or weaning of the young promotes involution of the mammary glands. Although use of rodent models has led to increased understanding of the initiation of programmed cell death following milk stasis, investigation of mammary involution in other species has provided information that highlights similarities and differences among species. The purpose of this review is to discuss the mammary involution and cell proliferation that occur during the nonlactating period between successive lactations in the predominant dairy species, i.e., cows, goats, and sheep. The time course and degree of mammary involution that occur in these animals differ markedly from those observed in the predominant research species, rodents.

Current management of dairy cows and goats results in significant overlap of lactation and pregnancy, such that these animals are typically pregnant when milking is terminated during late lactation. Thus, when milk stasis occurs, the mammogenic and lactogenic stimulation of pregnancy opposes stimuli for mammary involution. At this time, cows are most often well into the last trimester of pregnancy, and goats may be in the first days of pregnancy to the second trimester of pregnancy. Ewes are typically nonpregnant when milking is terminated. Differences in management of the reproduction/lactation cycles of dairy animals are due to the seasonal nature of goat and sheep reproduction in temperate climates, and the lengths of gestation and lactation for each species. Management approaches have evolved to maximize the profitability of milk production; in the process, the importance of a nonlactating period of limited involution between successive lactations has been determined for the cow.

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IMPACT OF A NONLACTATING PERIOD ON THE NEXT LACTATION

The nonlactating period prior to parturition in dairy animals is commonly referred to as the dry period. In cows, it has long been appreciated that the duration of the nonlactating interval is an important determinant of milk production in the subsequent lactation (1). Without a dry period, milk production may be reduced by 20%; and a dry period of 40–60 days is required for optimal production (2–4). The dry period appears to be important for reasons that center on the mammary gland, rather than on the nutritional status of the animal (2,5,6). Interestingly, a dry period between successive lactations was also found to be necessary for optimal lactation in rats (7). Litter weight gain was greater for rats that had undergone a four-day dry period than rats that had undergone no dry period or dry periods of 8, 12, or 16 days.

The determination of an optimal dry period for goats and sheep has received much less attention because the seasonal nature of the reproductive cycles in these species places constraints on manipulation of dry period length. Two investigations by Knight and coworkers addressed the importance of a dry period in goats. In the first experiment (8), lactating goats were mated during seasonal anestrus, subsequent to induction of ovulation with gonadotropin-releasing hormone. These goats entered the next lactation without an intervening dry period and milk production was found to be 12% less than for the previous lactation. Although these results are consistent with the importance of a dry period, they may have been confounded by an effect of season. Subsequently, these workers investigated the necessity for a dry period using a within goat, half-udder, design (9). One gland was milked during the prepartum period while the other was dried off 24 weeks (170 d) before parturition. There was no difference in milk production between glands. Indeed, at no stage of lactation was the milk yield of glands that had experienced a dry period numerically greater than that of the continuously milked glands, even though those glands were larger than continuously milked glands during the first few weeks after parturition. These data suggest that a dry period is not necessary for optimal milk production in goats. However, the 23–week dry period (three times the optimal length for cows) may have been too long and may have obviated the benefit of a dry period. Additionally, the effect of the half-udder experimental design should be considered. When one gland is no longer milked or secretion is inhibited by treatment with colchicine, milk production (10,11) and mammary growth (12) increase in a compensatory fashion in the lactating gland(s) within the same udder, and involution is partially inhibited in the nonlactating gland (13,14). Additionally, interactions of glands of differing lactational state within an udder on cell turnover and lactogenesis (the transition to lactation) remain uninvestigated. Because prepartum milking advances lactogenesis and milk production (15), milking one gland may conceivably advance lactogenesis and milk production in the other gland(s). Such premature milk synthesis without milk removal may cause a subsequent reduction in milk yield. Thus, it is plausible that milking one gland during the prepartum period inhibited the ability of the opposite gland to produce maximal quantities of milk during the subsequent lactation, or that milk production was increased in glands milked continuously when the opposite gland was dried off. Because compensatory milk production does not appear to occur in goats during late lactation (C. Wilde, personal communication), this likely was not a confounding factor in this study; howerver, pregnancy status, length of the dry period and other implications of a half-udder design may influence interpretation. Additional study is warranted to clarify the importance of a dry period in ruminants other than the cow.

CHANGES IN MORPHOLOGY AND MAMMARY CELL POPULATIONS AFTER CESSATION OF MILKING IN RUMINANTS

Morphology

To ascertain the extent of mammary involution after cessation of milking, it is necessary to evaluate morphological changes of the tissue combined with the evaluation of total mammary cell number. This section addresses morphological changes after cessation of milking and subsequent sections address changes in total cell number and specific cell populations.

Morphological changes that occur during the nonlactating period in dairy animals are less pronounced than those that occur during mammary involution in nonpregnant rats and mice. Morphological changes in dairy animals more closely reflect a change in the secretory state of the mammary gland rather than features characteristic of tissue regression. This is particularly evident during the dry period of dairy cows.

Figure 1 shows the morphology of bovine mammary tissue during the nonlactating period prior to parturition. The most evident morphological feature is the maintenance of alveolar structure throughout the 60–day prepartum period after milking has been terminated. Morphological changes in mammary tissue during the prepartum period were quantitatively evaluated (16) for cows that were dried off 60 days prepartum (Fig. 1) and for cows that were milked during this period. The percentage of tissue area occupied by mammary epithelium was not influenced by gestation day or by lactation status. Luminal area in mammary tissue from dry cows decreased to a minimum of 9.5% on day 35 prepartum (dry 25 days), which was lower than the equivalent value (21%) for lactating cows. Even at this time, lumina remained patent and contained a basophilic proteinaceous fluid. Thereafter, the luminal area in mammary tissue from dry cows increased and tended to be greater than that in lactating tissue at 7 days prepartum. These data are consistent with an initial absorption of milk during the dry period, followed by the initiation of synthetic and secretory activity leading to accumulation of mammary secretions during the final phases of lactogenesis. Furthermore, the area occupied by stromal elements was inversely related to luminal area. Stromal area in mammary tissue from dry cows increased to a maximum at 35 days prepartum and then decreased to a minimum at 7 days prepartum, at which time stromal area was less for dry cows than for lactating cows. In lactating cows, mammary stromal area did not change significantly during the prepartum period.

Others (17,18) have characterized the sequence of cytological changes in bovine mammary tissue over the weeks following cessation of milking. Within 24 hours of the last milking, there appeared to be a reduction in the fusion of secretory vesicles with the apical membrane of the mammary epithelial cells and a resulting accumulation of secretory vesicles and fat droplets within the alveolar cells. These changes apparently lead to vesicle fusion and formation of large stasis vacuoles that are apparent at the light microscopic level within 2 to 3 days. Accumulation of secretory products and vacuole formation are consistent with inhibition of milk secretion prior to an equivalent inhibition of milk synthesis (19). During the initial 48 hours, many cells display a decrease in cytoplasmic organelles involved in synthesis of milk; the Golgi apparatus is not distinguishable and the amount of rough endoplasmic reticulum is diminished within several days. By two weeks, most cells exhibit a marked reduction in apparent secretory capacity. However, the cells remain viable and retain a sufficient quantity of synthetic and metabolic organelles to allow for the synthesis of some secretory components, such as lactoferrin, during the dry period (20). About 30 days after cessation of milking, when the alveolar lumina are of minimal size, epithelial cells exhibit few vacuoles. During the two weeks before parturition the proportion of fully active cells increases, so that by 7 days prepartum nearly all alveolar cells display characteristics of cells poised for milk synthesis and secretion: cytoplasmic enlargement, accumulation of basal fat droplets and apical secretory vesicles (18).

Throughout the nonlactating period, the bovine mammary gland does not involute to the degree observed in other species (21). During mammary involution in rats and mice, epithelial cells undergo apoptosis and many of these apoptotic cells are shed into alveolar lumina (22,23), leading to the collapse and destruction of alveoli within 2 weeks (24). Neither sloughing of epithelial cells into the alveolar lumen nor detachment from the basement membrane were observed during the nonlactating period in dairy cows, suggesting that there was little net loss of cells during the dry period (16, 18, 19). Although a population of bovine mammary epithelial cells undergo apoptosis initiated by milk stasis (25), there is no significant tissue regression during the nonlactating period prior to parturition (16). Much of this species difference may be attributable to the effect of pregnancy on involution. Indeed, we have demonstrated that pregnancy inhibits involution of mouse mammary gland after forced weaning by a mechanism that involves the combined stimulation of cell proliferation and inhibition of apoptosis (Capuco, Li, and Furth, unpublished data). In contrast, pregnancy appears to promote apoptosis and gradual involution in the suckled, lactating murine gland (26).

Tatarczuch *et al.* (27) recently described the sequence of morphological events during mammary involution in nonpregnant sheep after weaning lambs on day 5 of lactation. Tissue was evaluated at 2, 4, 7, 15, 30, and 60 days of involution by light and electron microscopy. At 2 days, alveolar lumina were expanded, epithelial cells were flattened and some cells contained stasis vacuoles. Apoptosis within mammary tissue was manifested as epithelial cells with apoptotic nuclei or apoptotic bodies within cytoplasmic vacuoles, as well as presence of macrophages containing remnants of apoptotic epithelial cells. At 4 days of involution, the proportion of apoptotic cells was maximal. Whether

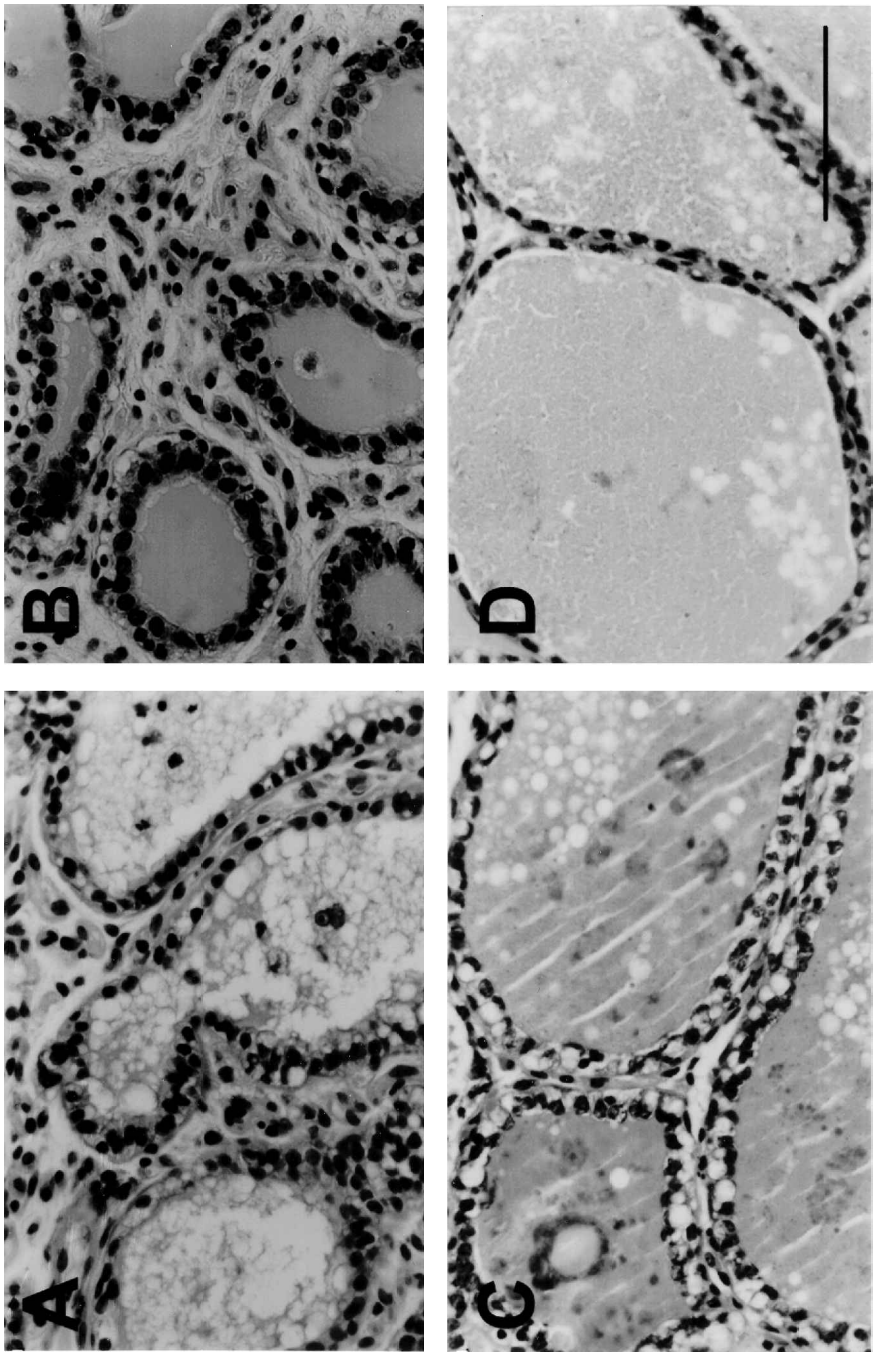

Fig. 1. Morphological appearance of bovine mammary glands during the preparturient, nonlactating (dry) period. Milking was terminated 60 days before expected parturition. Alveolar integrity was maintained during the nonlactating period. Several leukocytes are noted in the alveoli of panels A and B. (A) 53 days prepartum, 7 days dry; (B) 35 days prepartum, 25 days dry; (C) 20 days prepartum, 40 days dry; (D) 7 days prepartum, 53 days dry. Magnification is the same for all panels and is indicated by the 50-micron bar in panel D. [Quantitative morphology presented in Ref. (16).]

apoptosis is accompanied by cell proliferation and increased cell turnover has not been addressed. At day 4, alveolar distension was largely alleviated and most of the epithelial cells contained large vacuoles. By day 7, alveoli were reduced in size but intact, epithelial cells were highly vacuolated and there was a reduction in the rough endoplasmic reticulum and mitochondrial size. Macrophages containing cell remnants were evident in tissue at 15 days and these cells (identified by surface antigens) were prominent in many of the alveolar lumina, accounting for the highly vacuolated cells referred to as the cells of Donné. Epithelial cells maintained tight junction complexes. At 30 and 60 days after weaning, the tissue appeared to be fully involuted. Alveoli were still evident but contained very small lumina. Epithelial cells contained less rough endoplasmic reticulum, and a small Golgi apparatus, but retained many mitochondria and ribosomes. Myoepithelial cells were never observed to undergo apoptosis, nor were apoptotic figures observed in lactating tissue. During involution, the percentages of cells in apoptosis were 2.9, 3.6, 1.0, and 0.8% at 2, 4, 7 and 15 days of involution, respectively. It remains to be determined if the rate of apoptosis is significantly less than for mice, where 4.8% of epithelial cells were apoptotic 3 days after weaning (28).

There have been a limited number of histological examinations of involution in the goat. However, early events are similar to those in cows and ewes, and the time course of regression is similar to that in ewes (13,29). When milking was stopped the mammary gland underwent progressive involution. At 28 days, there were many intact alveoli, and increased stromal area. By 48 days, the gland had almost fully regressed to ducts with few alveoli. Although morphology during mammary involution has not been studied in pregnant goats, Fowler *et al.* demonstrated that the parenchymal volume of nonlactating glands did not decline more than that of lactating glands during the prepartum period of goats (9). However, because goats were not mated until several days after cessation of milking, early involution proceeded in the absence of a concomitant pregnancy. Results also may have been confounded by the use of a half-udder design, as described earlier.

Cell Populations

Accurate assessment of changes in mammary cell number during involution requires a combined evaluation of tissue morphology and total DNA content (30). Biochemical and histological measures were used to evaluate mammary involution and growth during the dry period in pregnant dairy cows (16). During this 60-day prepartum period, there was no evidence that a net loss of mammary cells (involution or regression) had occurred in nonlactating cows. At no time did mammary glands from nonlactating cows contain less total DNA or parenchymal mass than those from lactating cows (Fig. 2A). Furthermore, morphometric analysis demonstrated that tissue area occupied by mammary epithelium did not decline and that alveolar structures remained intact during the dry period (Fig. 1). Nonlactating and lactating cows entered the final week of gestation with an equal number of mammary cells (DNA). However, enumeration of cell types in tissue sections demonstrated that the proportion of total mammary cells that were epithelial was greater in nonlactating cows (83%) than in lactating cows (74%) one week before expected parturition (16).

Although mammary cell number did not differ between dry and lactating cows, the rate of [^{3}H]thymidine incorporation was 80% greater in mammary tissue from nonlactating cows than in mammary tissue from lactating cows (Fig. 2B) (16). Autoradiography indicated that increased incorporation of [^{3}H]thymidine was due to an increase in the percentage of mammary epithelial cells incorporating the nucleotide, most likely reflecting increased proliferation of epithelial cells within nonlactating glands. Because the number of mammary cells in nonlactating cows did not exceed

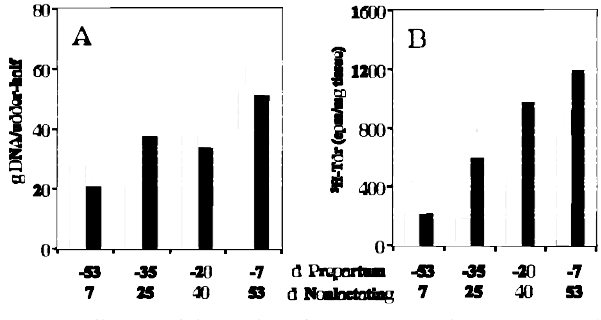

Fig. 2. Influence of the nonlactating state on total mammary DNA and [^{3}H]thymidine incorporation by mammary tissue during the prepartum period. Nonlactating cows had milking terminated 60 days before expected parturition and lactating cows were milked throughout the prepartum period. Day before expected parturition and days after cessation of milking are indicated on the *x* axis. Each bar represents the mean for 3–4 cows. (A) total DNA content of the udder-half; (B) *in vitro* incorporation of [^{3}H]thymidine by mammary tissue slices. □ lactating; ■ nonlactating. [Data from Ref. (16).]

that in lactating cows, the increased DNA synthesis was attributed to increased cell renewal or turnover. Similarly, Pitkow *et al.* (31) reported 60% greater turnover of mammary epithelial cells between first and second lactations when rats were permitted an intervening dry period of 7 to 8 days than when there was no nonlactating period. Thus, it has been suggested that a nonlactating period is important to enhance replacement of senescent mammary epithelial cells prior to the next lactation (16).

Cell Senescence

Milk production is a function of the number of mammary secretory cells and the secretory activity per cell. The concept that old or senescent mammary epithelial cells must be replaced prior to the successive lactation is predicated on two assumptions: 1) old cells do not secrete milk as efficiently or for as long as do young cells 2) old cells have reduced proliferative capacity so that the number of secretory cells declines prematurely during lactation. If these assumptions are true, then the persistency of lactation for cows that did not have a dry period of sufficient length should be less than that for cows with an appropriate dry period. However, this hypothesis is difficult to test and has not been addressed. Without sufficient cell renewal during lactation, milk production declines due to a decrease in the number of secretory cells and perhaps due to decreased milk production per cell. The decline in milk production during lactation in goats is solely due to a decrease in number of secretory cells (32), whereas declining cell number and activity per cell may both contribute to declining milk production as lactation advances in cows (Capuco, unpublished data). Cells that need to be replaced during the dry period may be those that are responsible for expanding and maintaining the number of mammary epithelial cells. Indeed, Paape and Tucker (7) demonstrated that mammary glands of rats that were not permitted a dry period had fewer cells at midlactation than glands of rats that were permitted a dry period of optimal length, although cell number did not differ at onset of the lactation.

Smith and colleagues have presented data pertaining to the nature of progenitor cells in the mouse mammary gland (33–35). They hypothesize that the murine mammary gland contains a population of mammary stem cells capable of generating all of the differentiated cells in the mammary gland, as well as progenitor cells with a more limited differentiation repertoire, with the ability to generate either ductal or alveolar cells (34,35). The ductal and lobular-alveolar progenitors have limited replication potential (34) and must be renewed from the stem cell population. Furthermore, these progenitor cells appear to undergo apoptosis during mammary involution (33), and it is reasonable to speculate that they must be renewed during the nonlactating period in order to maximize milk yield in the next lactation. If similar progenitor cells exist in bovine mammary tissue, we would hypothesize that the dry period is important to promote the renewal of this cell population (Fig. 3).

APOPTOSIS AND MAMMARY INVOLUTION

Following milk stasis, programmed cell death of mammary epithelium is initiated and provides for the turnover or reduction of mammary epithelial cells in dairy animals during the nonlactating period (25,27, 36). Apoptotic activity in the mammary tissue of ewes (27) appears to be initiated with a similar time course to that in rodents (22), beginning about 2 days post-weaning with a peak at about 4 days. The time course of apoptosis following milk stasis has not been reported for cows or goats; although 7 days after cessation of milking, apoptotic frequency was observed to be approximately 4% in bovine mammary tissue (25). Despite an apparent similarity in the induction of apoptosis, mammary involution proceeds more slowly in dairy animals than in rodents. Additionally, differ-

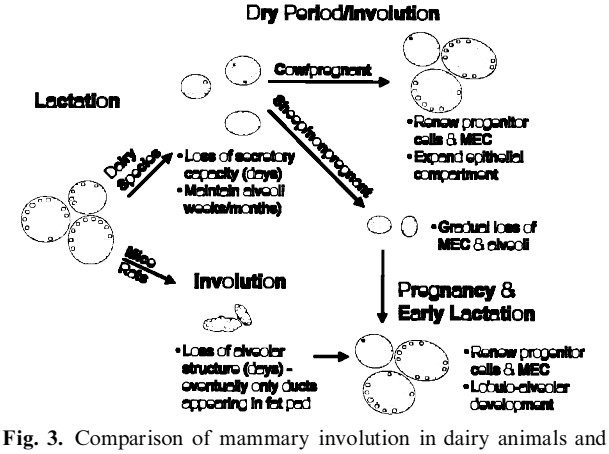

Fig. 3. Comparison of mammary involution in dairy animals and rodents. Involution in goats (not shown) is presumed to range between that of the cow and ewe, depending upon breeding management. MEC = mammary epithelial cells.

ences in tissue remodeling may be apparent, and possibly related to differences in absolute rates of apoptosis, simultaneous proliferation of cells, and to the endocrine status of the animal.

Factors that can influence the rate of mammary gland involution include systemic and local regulatory hormones/factors, pregnancy status, and stage of lactation when milk removal ceases. Involution in mice has been characterized as a two-stage process (24,37). During the first stage, mammary involution is triggered by local stimuli that initiate apoptosis, but the process can be reversed by reinitiating milk removal (24). However, the second stage of involution is irreversible and is characterized by activation of proteases that destroy the lobular-alveolar structure of the gland by degrading the extracellular matrix and basement membrane, as well as massive loss of alveolar cells. The second stage of involution can be inhibited by systemic glucocorticoids and progesterone (20,37) or by pregnancy (Capuco, Li, and Furth, unpublished data). Regulation of apoptosis in dairy species is described elsewhere in this issue (38).

In dairy cows, milking typically is terminated during late lactation, when milk yield is relatively low and the cow is in the final two months of a 9-month gestation. Under these circumstances it is not surprising that involution would proceed at a slow rate. Cessation of milking during late lactation probably results in slower involution than would be observed if milking was terminated during a high production period. Indeed, Quarrie *et al.* (26) demonstrated that involution in mice proceeds more slowly during natural weaning than during forced weaning on day 16 of lactation. As noted previously, concurrent pregnancy inhibits mammary involution in cows and may account for the maintenance of alveolar structure during the dry period (Figs. 1 and 3), consistent with the two stages of mammary involution noted in mice.

However, even without a concurrent pregnancy alveolar structure is partially maintained for several weeks in dairy animals (13,27,29) (Fig. 3). Consistent with the continued presence of intact alveoli, milk production of nonpregnant beef cows has been reinitiated four weeks after weaning (J. S. Stevenson, personal communication). A factor that may influence the rate of mammary involution is the possibility of concurrent cell proliferation. Clearly, proliferation occurs during the nonlactating period between successive lactations in dairy cows, but may also occur during involution in nonpregnant dairy animals. When suckling was permitted only on two glands of a beef cow increased [^{3}H]thymidine labeling of alveolar epithelial cells was evident in the nonsuckled glands 5 to 7 days after cessation of suckling (12). This synthesis of DNA may immediately precede apoptosis of the cell. Alternatively, it may signal proliferation of the mammary epithelium that inhibits mammary involution and permits restoration of milk synthesis during the early stages of milk stasis.

CONCLUSIONS

Involution of the mammary gland occurs at a slower rate and alveolar structure is maintained for a greater period of the involution process in dairy animals than in rodents (Fig. 3). In dairy cows, there is no detectable loss of mammary cells during the nonlactating period between successive lactations. However, a nonlactating period is nonetheless important for maximizing milk production in the ensuing lactation. Increased cell turnover during the nonlactating period may promote this effect by removing senescent epithelial cells and renewing a population of mammary cell progenitors.

Milk production efficiency can be increased by development and utilization of schemes that increase persistency of lactation and that minimize the duration of the nonlactating period. Knowledge of factors regulating apoptosis and cell proliferation during the nonlactating period should be instrumental in achieving this goal.

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